



A novel technique for aflatoxin M1 detoxification using chitin or treated shrimp shells: in vitro effect of physical and kinetic parameters on the binding stability

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Abstract

This study aimed to investigate the ability of chitin and heat-treated shrimp shells to bind aflatoxin M1 (AFM1) in liquid matrix. Several concentrations of chitin or shrimp shells (grinded and ungrinded) were incubated in AFM1-contaminated phosphate-buffered saline (PBS) at different incubation times. The stability of the formed adsorbent-AFM1 complex was also tested in milk at different incubation times and temperatures. The unbound AFM1 was quantified by HPLC. Thereby, the percentages of the initial bounded AFM1 varied between 14.29 and 94.74%. Interestingly, in milk, an increase in incubation time coupled with a decrease in temperature affected positively the amount of bounded AFM1 to chitin and negatively those bounded to ungrinded shells. Results also revealed a partial reversibility in the binding of AFM1 to these adsorbents. These findings provided strong evidence on ability of chitin or shrimp shells by-product to bind AFM1 in milk and in PBS.

Keywords Aflatoxin M1 · Chitin · *N*-acetylglucosamine · Shrimp shells · Milk · Binding

Introduction

Aflatoxins (AFs) are produced by several species of *Aspergillus* genera, mainly by *Aspergillus parasiticus* and *Aspergillus flavus* (Harper 2003). The production of AF on food depends on fungal growth at specific temperature and humidity conditions (Afsah-Hejri et al. 2013). After ingestion of AFB1-

contaminated feed, AFM1, a hydroxylated metabolite of aflatoxin B1, can be detected within 15 min in the animal blood and is then secreted in milk of lactated animals (Battacone et al. 2003; Fallah 2010). AFM1 remains stable at different stages of dairy processing such as pasteurization and sterilization (Fallah 2010). It is critical to mention that the International Agency for Research on Cancer (IARC) has classified AFM1 as a group 1 human carcinogen (Ostry et al. 2017). However, the maximum tolerable limit of AFM1 in milk varies between 0.05 µg/kg as settled by the European Union (EU) (European Commission 2006) and 0.5 µg/kg by Food and Drug Administration (FDA) (Food and Drug Administration—FDA 2005). Actually, reducing AF contamination in feed and food, by implementing prevention and control strategies, remains one of the best solutions in agro-food industry (Udomkun et al. 2017).

To date, several types of physical, chemical, and biological adsorbents have been studied for their ability to eliminate AF from food and feed (Nkana 1987; Jaynes and Zartman 2011). The use of different microorganisms including yeast and lactic acid bacteria (LAB) in order to bind AF in food have been extensively studied (Assaf et al. 2017; Faucet-Marquis et al. 2014; El Khoury et al. 2011). Hydrogen bonds and Van der Waals interactions were both previously identified to be

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implicated in the binding of AF (Shetty and Jespersen 2006; Yiannikouris et al. 2006). Both, polysaccharides and peptidoglycans, forming the cell wall of different microorganisms have been found to be involved in this binding (Shetty and Jespersen 2006; Kim et al. 2017). However, the amino sugar *N*-acetyl-D-glucosamine (GlcNAc), a key component of numerous cell walls including fungi, bacteria, and yeast (Chen et al. 2010), is also found in the exoskeleton of crustaceans by its polymer “chitin” (Xu et al. 2008; Khan et al. 2017).

Chitin (C₈H₁₃O₅N)_n is the second most abundant natural biopolymer of GlcNAc and contains acetamide groups (–NHCOCH₃) (Dutta et al. 2004). Furthermore, chitin is indigestible (Stengler 2005), insoluble in water, and does not dissolve in standard polar or non-polar solvents (Dutta et al. 2004). Due to its excellent properties including biocompatibility and non-toxicity, chitin has numerous applications in different fields such as material science, agriculture, microbiology, water treatment, drug delivery, tissue engineering, bionanotechnology, and food technology (Khoushab and Yamabhai 2010). Furthermore, chitin has a rigid crystalline structure with strong intra- and intermolecular hydrogen bonding (Minke and Blackwell 1978; González et al. 2000). In the USA, the use of chitin and its deacetylated form chitosan in food and food supplements are FDA approved since 1983 (Dossey et al. 2016; GRAS Notices n.d.). In that sense, attention was given to these chitinous polymers in food application and food research. A wide range of applications of these biopolymers was offered including bioconversion in food production (Paul and Raymond 1978; Shahidi and Synowiecki 1991), food preservation (Muzzarelli et al. 2012), and biodegradable film formation (Averous 2009). In addition, these polysaccharides can be used as additives for clarification, deacidification, texturization, emulsification, and stabilization of fruit juices, beverages, and dairy products (GRAS Notices n.d.; Patent CA2547760A1 n.d.).

Chitin is also a primary component of crustacean exoskeleton, specifically shrimp shells that contain 30–40% chitin, 35% proteins, 20–30% calcium carbonate, and 5–10% lipids (Venugopal 2016). Nowadays, between 6 and 8 million tons of crab, shrimp, and lobster shells waste are yearly produced worldwide including 1.5 million tons in southeast Asia alone (Yan and Chen 2015). In less developed countries, the potential values of the shell waste are ignored and usually discarded in a landfill or dumped in the sea (Yan and Chen 2015). Nevertheless, several food industries benefit from the inclusion of crustacean shells in their products. These foodstuffs include sauce made from shrimp, crab, and lobster by-products (Kim et al. 2003; Lee et al. 2015), in addition to fish cakes supplemented with shrimp powder (Seo and Cho 2012). Furthermore, a study on supplementing the Appenzeller Swiss cheese with shrimp powder conducted by Lee et al. (2015) revealed an absence of any significant alteration in the quality of cheese after shell inclusion. Therefore, food industries are profiting from the added value of the bioactive compounds of

crustacean shells such as chitin and chitosan. These benefits include strengthening the immune system, reducing blood pressure, anti-aging activity, suppressing cancer cell proliferation, promoting the proliferation of beneficial intestinal bacteria, and improving liver function and glycemic control (Lee et al. 2015). In this work, we aimed to evaluate and compare the ability of different concentrations of chitin or heat-treated shrimp shells to bind AFM1 in PBS and in milk. The stability of the formed complex with AFM1 was studied after repetitive washes. Furthermore, the effect of different incubation times and temperatures on AFM1 binding was also studied.

Materials and methods

Aflatoxin M1 binding assay

Preparation of AFM1-contaminated PBS and milk

An AFM1 standard (10 µg/mL) suspended in acetonitrile was obtained from Sigma (St. Louis, MO, USA). Two contamination levels of PBS and milk with a concentration of 5 and 2 µg/L were prepared by diluting the AFM1 stock solution in PBS (pH 7.4, 0.05% Tween 20, *v/v*) and in whole UHT milk (purchased from the local market). Prior to their use, the acetonitrile was evaporated from the solutions by heating in a water bath at 80 °C for 10 min. In addition, no AFM1 was detected in the tested milk samples before the binding procedure.

Preparation of adsorbents

Chitin from shrimp shells was obtained from Sigma-Aldrich (St. Louis, MO, USA). Fresh shrimps were purchased from local market. The organic part attached to the shrimp shells was completely removed, and shells were washed with sterile distilled water. Shrimp shells were dried under ambient air flow. For grinded samples, a domestic blender was used to pulverize the shells. Microtubes (Eppendorf AG, Hamburg, Germany) of 1.5 mL were used for chitin and shrimp shells with quantities less or equal to 0.05 and 0.1 g, respectively. Higher quantities were suspended in 14 mL tubes (Falcon, Corning Inc., NY, USA). Chitin and shrimp shells were washed five times with PBS and impurities were removed. Shells were then suspended in PBS and heated in a water bath at 90 °C for 1 h. After centrifugation at 3000×*g* for 10 min, all sample supernatants were discarded.

Binding procedure

Chitin or shrimp shells were suspended in 1 mL of AFM1 contaminated PBS or whole UHT. A pipetting of suspensions was conducted for 30 s prior to incubation. All suspensions in PBS were then incubated at 37 °C for 30 min or 24 h. Milk

suspensions were even incubated for 30 min at 37 °C or left for 24 h at 24 °C. After incubation, the suspensions were centrifuged at 3000×g for 10 min and supernatant was removed. The unbound AFM1 was then quantified by HPLC. Negative control (only chitin or shrimp shells suspended in PBS/Milk) and positive control (only AFM1 in PBS/milk) were also incubated.

Effect of chitin and shrimp shell concentration on AFM1 binding

The concentration effects of both chitin and shrimp shells were studied in milk and PBS. For tests in PBS, an AFM1 concentration of 5 and 2 µg/L was applied to different concentrations of chitin and grinded and ungrinded shrimp shells. The AFM1 concentration was kept constant at 2 µg/L for binding tests in milk. Different chitin concentrations were used: 0.004, 0.02, and 0.05 g/mL in PBS, and in parallel, 0.05 and 0.4 g/mL were suspended in milk. Furthermore, concentrations of 0.02, 0.1, and 0.15 g/mL of grinded shrimp shells were used for AFM1 binding in PBS, in addition to 0.15 and 0.7 g/mL in milk. For ungrinded shrimp shells, concentrations of 0.02 and 0.25 g/mL were used for binding tests contaminated solution, samples were incubated at different in PBS in addition to 0.25 and 0.7 g/mL in milk. After being suspended in AFM1-incubation times and temperatures.

Effect of incubation times and temperatures on AFM1 binding

After conducting a preliminary study, two different incubation temperatures and times were undertaken and their effects on AFM1 binding were elucidated. Despite the used concentration of AFM1 (2 µg/L and 5 µg/L), chitin concentration of 0.004, 0.02, and 0.05 g/mL were incubated with AFM1 in PBS for 30 min at 37 °C. In addition, shrimp shells with concentrations of 0.02, 0.1, and 0.15 g/mL (grinded shells) and of 0.02 and 0.25 g/mL (ungrinded shells) were similarly incubated with AFM1 in PBS at same conditions. On the other hand, chitin concentration of 0.05 g/mL and shells with a concentration of 0.15 g/mL (grinded) and 0.25 g/mL (ungrinded) were incubated in PBS with AFM1 for 24 h at 37 °C.

Binding tests in milk were divided in two different groups. The first group of samples were incubated with AFM1 for 30 min at 37 °C using chitin concentration of 0.05 and 0.4 g/mL. Similarly, shrimp shells with concentration of 0.15 and 0.7 g/mL (grinded) in addition to 0.25, and 0.7 g/mL (ungrinded) were incubated at the same conditions. The second group of samples was incubated with AFM1 for 24 h using chitin concentration of 0.05 g/mL and shell concentration of 0.15 g/mL (grinded) and 0.25 g/mL (ungrinded). Accordingly, this group of samples were incubated at 24 °C (24 h) to avoid UHT milk spoilage

that may appear when incubated for long period at higher temperature (Richards et al. 2014).

Effect of washes on AFM1/chitin and AFM1/shrimp shells complexes

After performing the binding assay as previously described, the effect of washes on chitin/AFM1 and shrimp shells/AFM1 complex were studied. The pellets of chitin and shrimp shells incubated in AFM1-contaminated solution were washed by suspending in 1 mL of the same solution (PBS or milk). After each wash, suspensions were kept for 10 min at room temperature. Samples were then centrifuged at 3000×g for 10 min and supernatants were collected. The washing step was repeated five times and residual AFM1s were quantified by HPLC.

Residual AFM1 quantification

After incubation of AFM1 with chitin and shrimp shells (grinded and ungrinded) separately, the residual AFM1 were quantified using reverse-phase HPLC (Waters 2690®, Waters Corp., MA, USA). The HPLC was coupled with a fluorescence detector (Waters 2475®, Waters Corp., MA, USA) and a (Supelco Discovery®, Sigma-Aldrich Co., MO, USA) C18 column (250 × 4.6 mm I.D, 5 µm particle diameter) fitted with a C18 guard column (Supelco Supelguard®, Sigma-Aldrich Co., MO, USA). The residual AFM1 in PBS samples was determined using a reverse-phase HPLC method (without AFM1 extraction), as described by Pierides et al. (2000) with minor modifications. The quantification of AFM1 in milk samples was performed using immunoaffinity columns (R-Biopharm, Darmstadt, Germany) for clean-up and in accordance with the instructions supplied by the equipment manufacturer. Samples were performed according to AOAC International Official Method 2000.08 (AOAC 2005). AFM1 in milk was recovered in a mobile phase composed of water/acetonitrile/methanol (68/24/8; vol/vol/vol). The HPLC mobile phase was composed of HPLC water/acetonitrile/methanol (68/24/8; vol/vol/vol), and its flow rate was fixed at 1 mL/min. The injection volume was 20 µL for samples tested in PBS and 100 µL for milk samples. Fluorescence detection was used with an excitation and emission wavelengths of 360 and 430 nm, respectively. The retention time for AFM1 was 9.4 min. A calibration curve was established using different concentrations of AFM1 standard (Sigma Aldrich, MO, USA) ranging from 0.03 to 10 µg/L.

Statistical analysis

All tests were done in triplicate. To identify significantly different results, two-way ANOVA was conducted using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). The results with a $P < 0.05$ were considered statistically significant.

Results

Complex binding stability in PBS

Different concentrations of chitin and shrimp shells were incubated for 30 min and 24 h in AFM1-contaminated PBS, and the stability of the formed complexes was studied. The results are presented in Tables 1 and 3 with their standardized effects in Tables 2 and 4 respectively.

Binding stability at different adsorbent concentrations during an incubation time of 30 min

Results showed that AFM1 binding ability varied according to different adsorbent concentrations and different AFM1 contamination level in PBS. In PBS contaminated with AFM1 (5 µg/L), the increasing chitin concentration from 0.004 to 0.02 g/mL significantly increased ($P < 0.05$) the initial binding of AFM1 from 17.16 to 35.30%, respectively (Tables 1 and 2). Similarly, increasing grinded shrimp shell concentration from 0.02 to 0.1 g/mL resulted in a significant increase ($P < 0.05$) in the initial binding from 21.54 to 88.12%, respectively (Tables 3 and 4). After washing, the final binding of AFM1 also increased ($P < 0.05$) from 12.61% (0.004 g/mL) to 27.15% (0.02 g/mL) with chitin and from 10.04% (0.02 g/mL) to 67.07% (0.1 g/mL) with grinded shells. For the same concentration of grinded and ungrinded shrimp shells (0.02 g/mL), the highest significant ($P < 0.05$) amount of bounded AFM1 was that attained with grinded shells. Hence, a binding increase from 14.29% (ungrinded) to 21.54% (grinded) before washing and from 4.31% (ungrinded) to 10.04% (grinded) after five washes was observed.

While decreasing the AFM1 concentration to 2 µg/L in PBS medium and after increasing chitin and shrimp shell concentration, a significant difference in the initial binding ($P < 0.05$) was observed. The initial yield amount of bounded AFM1 to chitin has increased 24.43% (from 17.16 to 41.59%) by increasing its concentration from 0.004 to 0.05 g/mL (Tables 1 and 2). Similarly, when increasing the concentration of grinded shrimp shells from 0.02 to 0.15 g/mL, this binding significantly increased ($P < 0.05$) of 73.2%

(from 21.54 to 94.74%) (Tables 3 and 4). Furthermore, an increase ($P < 0.05$) of 71.24% (from 14.29 to 85.53%) in AFM1 initial binding has resulted when increasing ungrinded shell concentration from 0.02 to 0.25 g/mL. In the same manner, this significant increase ($P < 0.05$) in the initial binding of AFM1 to these adsorbents was also observed after the completion of five washes (Tables 3 and 4).

Binding stability at different incubation times in PBS contaminated with 2 µg/L AFM1

When increasing the incubation time of AFM1 with chitin and shrimp shells from 30 min to 24 h, the binding difference in some experiments was quite significant (Tables 1, 2, 3, 4). For the same chitin concentration of 0.05 g/mL, the initial binding of AFM1 significantly increased ($P < 0.05$) from 41.59% (30 min) to 54.45% (24 h) (Tables 1 and 2). Similarly, the binding after five washes was also significantly different ($P < 0.05$) and increased from 30.27% (30 min) to 39.39% (24 h). Moreover, the initial binding of AFM1 to 0.25 g/mL of ungrinded shrimp shells showed a significant binding yield increase ($P < 0.05$) from 85.53% (30 min) to 93.07% (24 h) (Tables 3 and 4). After washing, AFM1 binding also increased ($P < 0.05$) from 42.35% (30 min) to 73.07% (24 h). On the other hand, after 24 h of incubation of AFM1 with grinded shrimp shells (0.15 g/mL), no significant difference was observed ($P > 0.05$) when compared to 30 min (Tables 3 and 4).

Binding stability after successive washes in PBS contaminated with 2 µg/L AFM1

The effect of successive washing steps on the binding of AFM1 to chitin and shrimp shells after 30 min and 24 h of incubation is presented in Fig. 1.

The first two consecutive washes showed a significant decrease ($P < 0.05$) of 11.32% (30 min) and 15.06% (24 h) after incubation of AFM1 with chitin. Similarly, a significant decrease ($P < 0.05$) in AFM1 binding was observed when washing grinded shrimp shells. Accordingly, a decrease of 32.69% (30 min) after four washes and 26.87% (24 h) after three washes was shown. In the same manner, when using

Table 1 Effect of chitin concentrations and incubation times on the binding of AFM1 (2 and 5 µg/L) in PBS (37 °C) before (initial) and after (final) five washes

Adsorbent	Concentration (g/mL)	AFM1 (µg/L)	Incubation time	Initial binding (%)	Final binding (%)
Chitin	0.004	5	30 min	17.16 ± 1.37a	12.61 ± 1.45b
	0.02	5	30 min	35.30 ± 2.48c	27.15 ± 2.51d
	0.05	2	30 min	41.59 ± 2.24e	30.27 ± 2.31f
	0.05	2	24 h	54.45 ± 1.89g	39.39 ± 1.94h

Results are the average ± SD for triplicate samples. Lowercase letters represent the percentage of binding at specific concentrations of chitin, AFM1, and incubation time

Table 2 Statistical data of the effects of chitin concentrations and incubation times on the binding of AFM1 (2 and 5 µg/L) in PBS (37 °C) before (initial) and after (final) five washes

Hypothesis	Comparisons of datasets	Result
H1: The means of initial and final bindings are not equal.	a-b, c-d, e-f, g-h	Accepted ($P < 0.05$)
H2: The means of initial bindings at same incubation time and AFM1 concentration with different adsorbent concentrations are not equal.	a-c	Accepted ($P < 0.05$)
H3: The means of final bindings at same incubation time and AFM1 concentration with different adsorbent concentrations are not equal.	b-d	Accepted ($P < 0.05$)
H4: The means of initial bindings at same adsorbent and AFM1 concentrations with different incubation times are not equal.	e-g	Accepted ($P < 0.05$)
H5: The means of final bindings at same adsorbent and AFM1 concentrations with different incubation times are not equal.	f-h	Accepted ($P < 0.05$)
H6: The means of initial bindings at same incubation time with different AFM1 and adsorbent concentrations are not equal.	a-e, c-e	Accepted ($P < 0.05$)
H7: The means of final bindings at same incubation time with different AFM1 and adsorbent concentrations are not equal.	b-f d-f	Accepted ($P < 0.05$) Rejected ($P > 0.05$)

Lowercase letters represent the percentage of binding at specific concentrations of chitin, AFM1, and incubation time as defined in Table 1

ungrinded shells, a significant binding decrease ($P < 0.05$) of 43.18% (30 min) was observed after four washes and 20% (24 h) after three washes.

Complex binding stability in milk

Chitin and shrimp shells were incubated with AFM1 (2 µg/L) for 30 min (37 °C) and 24 h (24 °C). The stability of the formed complex in milk solution was studied. The results were highlighted in Tables 5 and 7 with their standardized effects in Tables 6 and 8, respectively.

Binding stability at different adsorbent concentrations and after several washes

In order to study the stability of the formed complex in milk, AFM1 (2 µg/L) was incubated with different concentrations of chitin (Tables 5 and 6) and shrimp shells (Tables 7 and 8) for 30 min at 37 °C.

When increasing chitin concentration from 0.05 to 0.4 g/mL, the initial binding of AFM1 has significantly increased ($P < 0.05$) from 55.99 to 84.57% (28.58%) (Tables 5 and 6). Similarly, an increase in grinded shell concentration from 0.15 to 0.7 g/mL has increased ($P < 0.05$) this binding from 72.55 to 84.82% (12.27%) (Tables 7 and 8). Furthermore, after increasing the concentration of ungrinded shrimp shells from 0.25 to 0.7 g/mL, the initial amount of bounded AFM1 increased ($P < 0.05$) from 77.44 to 88.59% (11.15%) (Tables 7 and 8).

After five washes, the binding of AFM1 to grinded shells showed a significant increase ($P < 0.05$) from 50.56 to 64.12% (13.56%), when increasing its concentration from 0.15 to 0.7 g/mL (Tables 7 and 8). Similarly, increasing the concentration of ungrinded shells from 0.25 to 0.7 g/mL increased significantly ($P < 0.05$) the final amount of bounded AFM1 from 52.49 to 63.13% (10.64%) (Tables 7 and 8). On the other hand, despite the chitin concentration increase from 0.05 to 0.4 g/mL, no significant ($P > 0.05$) binding difference was observed (Tables 5 and 6).

Table 3 Effect of treated shrimp shell concentration and incubation times on the binding of AFM1 (2 and 5 µg/L) in PBS (37 °C) before (initial) and after (final) five washes

Adsorbent	Concentration (g/mL)	AFM1 (µg/L)	Incubation time	Initial binding (%)	Final binding (%)
Grinded shrimp shells	0.02	5	30 min	21.54 ± 1.35a	10.04 ± 1.51b
	0.1	5	30 min	88.12 ± 2.33c	67.67 ± 2.42d
	0.15	2	30 min	94.74 ± 3.01e	62.05 ± 3.10f
	0.15	2	24 h	91.71 ± 3.12g	64.84 ± 3.08h
Ungrinded shrimp shells	0.02	5	30 min	14.29 ± 1.54i	4.31 ± 1.10j
	0.25	2	30 min	85.53 ± 2.56k	42.35 ± 2.91l
	0.25	2	24 h	93.07 ± 2.75m	73.07 ± 2.82n

Results are the average ± SD for triplicate samples. Lowercase letters represent the percentage of binding at specific concentrations of shells, AFM1 and incubation time

Table 4 Statistical effects of treated shrimp shell concentration and incubation times on the binding of AFM1 (2 and 5 µg/L) in PBS (37 °C) before (initial) and after (final) five washes

Hypothesis	Comparisons of datasets	Result
H1: The means of initial and final bindings are not equal.	a-b, c-d, e-f, g-h, i-j, k-l, m-n	Accepted ($P < 0.05$)
H2: The means of initial bindings at same incubation time and AFM1 concentration with different adsorbent concentrations are not equal.	a-c	Accepted ($P < 0.05$)
H3: The means of final bindings at same incubation time and AFM1 concentration with different adsorbent concentrations are not equal.	b-d	Accepted ($P < 0.05$)
H4: The means of initial bindings at same adsorbent and AFM1 concentrations with different incubation times are not equal.	k-m e-g	Accepted ($P < 0.05$) Rejected ($P > 0.05$)
H5: The means of final bindings at same adsorbent and AFM1 concentrations with different incubation times are not equal.	l-n f-h	Accepted ($P < 0.05$) Rejected ($P > 0.05$)
H6: The means of initial bindings at same incubation time with different AFM1 and adsorbent concentrations are not equal.	a-e, c-e, i-k	Accepted ($P < 0.05$)
H7: The means of final bindings at same incubation time with different AFM1 and adsorbent concentrations are not equal.	b-f, j-l d-f	Accepted ($P < 0.05$) Rejected ($P > 0.05$)

Lowercase letters represent the percentage of binding at specific concentrations of shells, AFM1, and incubation time as defined in Table 3

Binding stability at different incubation times and temperatures

After increasing the incubation time from 30 min to 24 h and decreasing the incubation temperature from 37 to 24 °C respectively, the stability of the formed complex was assessed (Tables 5, 6, 7, and 8).

The increase of incubation time from 30 min to 24 h has significantly increased ($P < 0.05$) the initial amount of bounded AFM1 to chitin (0.05 g/mL) from 55.99 to 73.73%

respectively (Tables 5 and 6). Similarly, after five washes, this initial binding increased ($P < 0.05$) from 37.87% (30 min) to 60.03% (24 h).

Conversely, after increasing the incubation time from 30 min to 24 h, the binding of AFM1 to grinded shrimp shells (0.15 g/mL) did not show any significant difference ($P > 0.05$) (Tables 7 and 8). Hence, this difference in AFM1 binding after 30 min (37 °C) and 24 h (24 °C) of incubation was quite similar before (from 72.55 to 72.26%; $P > 0.05$) and after five washes (from 50.56 to 49.85%; $P > 0.05$) respectively.

Fig. 1 Effect of successive washes on AFM1/chitin and AFM1/shell complex binding stability in PBS. Binding was determined after incubation of AFM1 (1 mL and 2 µg/L) with chitin and shrimp shells for 30 min and 24 h at 37 °C. The formed complexes were washed five times with 1 mL of PBS and the unbound AFM1 was quantified. The results are mean values from three experiments; *Indicates a significant difference ($P < 0.05$) between each step and its previous at the same incubation time. Error bars represent the SD (standard deviation)

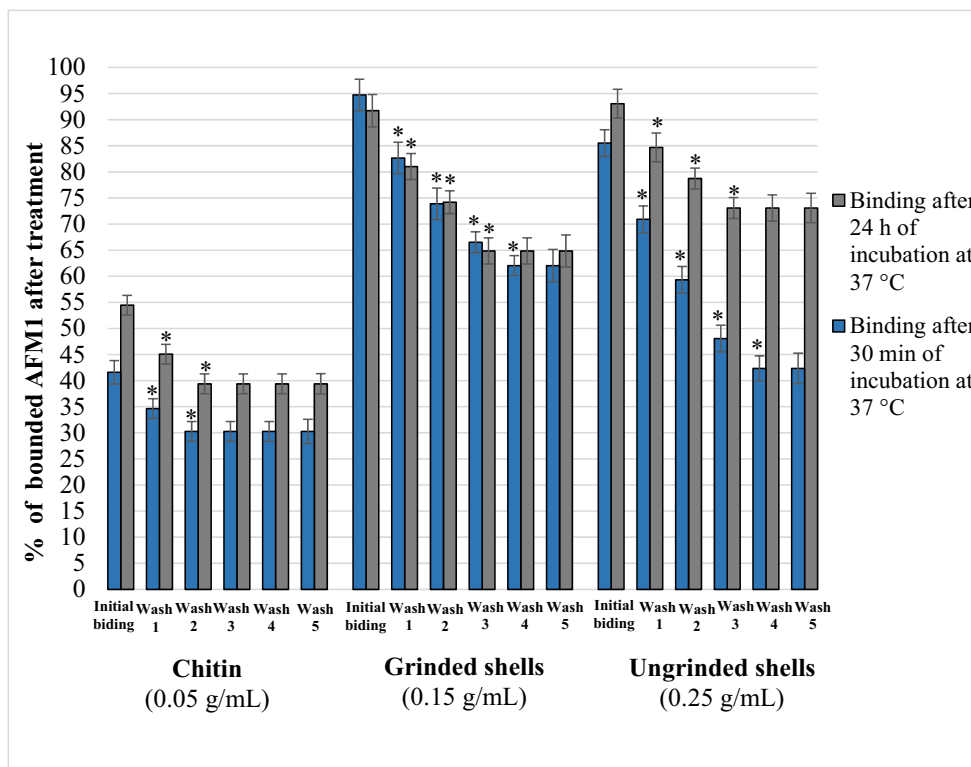


Table 5 Effect of chitin concentrations, incubation times, and temperatures on the binding of AFM1 (2 µg/L) in milk before (initial) and after (final) five washes

Adsorbent	Concentration (g/mL)	Incubation time	Incubation temperature	Initial binding (%)	Final binding (%)
Chitin	0.05	30 min	37 °C	55.99 ± 3.63a	37.87 ± 3.92b
	0.05	24 h	24 °C	73.73 ± 3.96c	60.03 ± 3.51d
	0.4	30 min	37 °C	84.57 ± 3.84e	34.05 ± 3.90f

Results are the average ± SD for triplicate samples. Lowercase letters represent the percentage of binding at specific concentrations of chitin, incubation time, and incubation temperature

The incubation of AFM1 with 0.25 g/mL of ungrinded shrimp shells for 24 h at 24 °C resulted in a significant ($P < 0.05$) decrease in the binding of AFM1 compared to when incubated for 30 min at 37 °C (Tables 7 and 8). Moreover, AFM1 binding decreased respectively from 77.44 to 66.57% before washing and from 52.49 to 36.10% after five washes.

Discussion

Results of Table 1 showed an increase in the initial binding of AFM1 after increasing chitin and shell concentration during an incubation time of 30 min. It could be speculated that the increase in chitin and shell concentration may have caused the formation of additional electrostatic bonds with AFM1 such as Van der Waals and hydrogen bonds (Shetty and Jespersen 2006; Yiannikouris et al. 2006). Furthermore, for the same concentration of grinded and ungrinded shrimp shells (0.02 g/mL), binding was significantly higher ($P < 0.05$) with grinded shells (Tables 3 and 4). This difference between grinded and ungrinded shells may be explained by the distribution of powdered shrimp shells in PBS, resulting in a higher contact with AFM1. In addition, the grinding step has possibly increased the specific surface of shrimp shells, causing an increase in the exposure of different shell layers as epi-, exo-, and endocuticle to AFM1 (Xu et al. 2013). Thus, this binding increase is possibly due to the decrease of AFM1 concentration from 5 to 2 µg/L, resulting in an increase in the amount of

bound AFM1. In addition, keeping the contaminated PBS volume fixed and increasing the adsorbent concentration may have increased the exposure of AFM1 to these binders.

The results of binding stability at different incubation times in PBS contaminated with AFM1 (2 µg/L) shown in Tables 1 and 2 indicate a positive effect of incubation time on the binding of AFM1 to chitin in PBS. Thus, incubation time may have increased the contact between AFM1 and chitin particles. Similarly, the binding of AFM1 to ungrinded shrimp shells (0.25 g/mL) showed a significant binding increase ($P < 0.05$) (Tables 3 and 4). This increase of 30.72% after incubation for longer period (24 h) may reveal higher bonding strength of AFM1 to ungrinded shells. Roer and Dillaman (1984) while studying the structure and calcification of crustacean cuticle mentioned the composition of the three layers forming this cuticle. The epicuticle, which is the outermost layer, is mainly composed of minerals that are present as spherulitic calcite islands and surrounded by a lipoprotein matrix. Beneath the epicuticle, the exo- and endocuticle are located and consists of chitin-protein fibers embedded with mineral-consisted crystals. A possible loose binding of some AFM1 to epicuticle components may have appeared when incubated with AFM1 for short period of time (30 min). Therefore, when incubated for longer period (24 h), higher amounts of AFM1 pass through the epicuticle and bind to components of exo- and endocuticle layers as chitin. In addition, the presence of cuticles may have increased the binding stability of AFM1 during washing steps. We can hypothesize that the binding of AFM1 to grinded

Table 6 Statistical effects of chitin concentrations, incubation times, and temperatures on the binding of AFM1 (2 µg/L) in milk before (initial) and after (final) five washes

Hypothesis	Comparisons of datasets	Result
H1: The means of initial and final bindings are not equal.	a-b, c-d, e-f	Accepted ($P < 0.05$)
H2: The means of initial bindings at same adsorbent concentrations with different incubation times and temperatures are not equal.	a-c	Accepted ($P < 0.05$)
H3: The means of final bindings at same adsorbent concentrations with different incubation times and temperatures are not equal.	b-d	Accepted ($P < 0.05$)
H4: The means of initial bindings at same incubation time and temperature with different adsorbent concentration are not equal.	a-e	Accepted ($P < 0.05$)
H5: The means of final bindings at same incubation time and temperature with different adsorbent concentration are not equal.	b-f	Rejected ($P > 0.05$)

Lowercase represent the percentage of binding at specific concentrations of chitin, incubation time, and incubation temperature as defined in Table 5

Table 7 Effect of treated shrimp shell concentration, incubation times, and temperatures on the binding of AFM1 (2 µg/L) in milk before (initial) and after (final) five washes

Adsorbent	Concentration (g/mL)	Incubation time	Incubation temperature	Initial binding (%)	Final binding (%)
Grinded shrimp shells	0.15	30 min	37 °C	72.55 ± 3.14a	50.56 ± 3.22b
	0.15	24 h	24 °C	72.26 ± 2.55c	49.85 ± 3.63d
	0.7	30 min	37 °C	84.82 ± 3.03e	64.12 ± 3.62f
Ungrinded shrimp shells	0.25	30 min	37 °C	77.44 ± 3.10 g	52.49 ± 3.94h
	0.25	24 h	24 °C	66.57 ± 2.54i	36.10 ± 3.62j
	0.7	30 min	37 °C	88.59 ± 3.25k	63.13 ± 3.34l

Results are the average ± SD for triplicate samples. Lowercase letters represent the percentage of binding at specific concentrations of shells, incubation time and incubation temperature

shrimp shells in PBS is quite fast and does not require high incubation period to allow binding additional amounts of AFM1. Nevertheless, this slight decrease ($P > 0.05$) in the initial binding (Tables 3 and 4) of AFM1 (from 94.74 to 91.71%) may reveal a disruption of some electrostatic bonds after increasing the incubation time. Conversely, after five washes, a slight increase ($P > 0.05$) in the final binding (from 62.05 to 64.84%) was observed. Therefore, increasing the incubation time from 30 min to 24 h has led to a possible strengthening of the formed bonds.

The complex stability after successive washes in PBS revealed a partial reversibility of AFM1 binding to chitin and shrimp shells (Fig. 1). This partial reversibility is an indicator of the presence of non-covalent bonding that may be disrupted after successive washes. Moreover, washing AFM1/chitin and shrimp AFM1/shell complexes with PBS might have released back into solution amounts of unbound AFM1 stacked among chitin particles and shrimp shells (Assaf et al. 2017).

The AFM1 binding in milk when using different adsorbent concentration has significantly increased ($P < 0.05$) after increasing their concentrations (Fig. 2). Therefore, this high binding affinity revealed the potential ability of these adsorbents to bind additional amounts of AFM1 for a same volume of contaminated milk. The absence of a significant binding increase

($P > 0.05$) of chitin despite its concentration increase from 0.05 to 0.4 g/mL was noticeable. Thus, this absence of a significant difference may be explained by the high decrease ($P < 0.05$) from 84.57 to 34.05% (50.52%) in the amount of AFM1 bound to chitin (0.4 g/mL) after five washes. We could speculate that the sharp decrease in AFM1 binding may be due to an increase of friction between chitin particles causing a disruption of the formed electrostatic bonds with AFM1. In addition, due to the increase in chitin concentration, a higher retention of unbound AFM1 among chitin particles may appear. Accordingly, this massive binding decrease was not observed when using shrimp shells due to the presence of different shell layers (Xu et al. 2013) that may increase the binding stability of AFM1 from adverse external forces. In contrast, different strategies can be adopted to possibly increase this binding. Thus, by increasing the fixed inoculum volume (1 mL) or decreasing the used chitin concentration (0.4 g/mL), a higher binding of AFM1 with chitin in milk may take place.

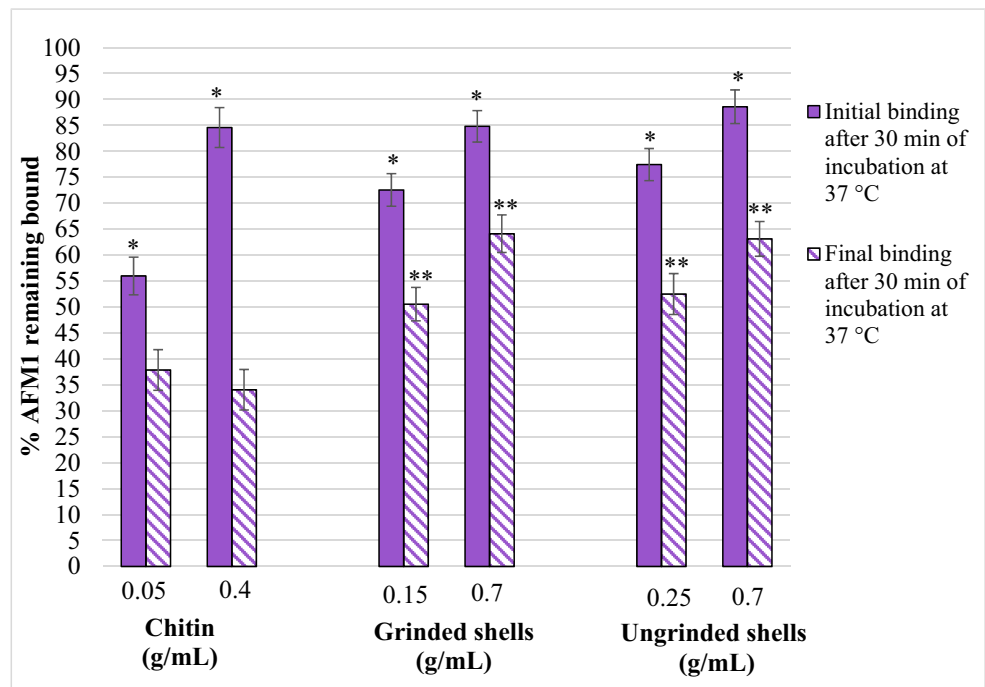
The binding stability of AFM1 to chitin and shrimp shells in milk solution was also assessed (Fig. 3). The incubation time increase and temperature decrease appear to have a positive effect on the binding of AFM1 to chitin. In addition, the probable absence of incubation time and temperature effect on AFM1 binding may reveal a rapid and stable binding of

Table 8 Statistical effects of treated shrimp shell concentration, incubation times, and temperatures on the binding of AFM1 (2 µg/L) in milk before (initial) and after (final) five washes

Hypothesis	Comparisons of datasets	Result
H1: The means of initial and final bindings are not equal.	a-b, c-d, e-f, g-h, i-j, k-l	Accepted ($P < 0.05$)
H2: The means of initial bindings at same adsorbent concentrations with different incubation times and temperatures are not equal.	g-i	Accepted ($P < 0.05$)
	a-c	Rejected ($P > 0.05$)
H3: The means of final bindings at same adsorbent concentrations with different incubation times and temperatures are not equal	h-j	Accepted ($P < 0.05$)
	b-d	Rejected ($P > 0.05$)
H4: The means of initial bindings at same incubation time and temperature with different adsorbent concentration are not equal.	a-e, g-k	Accepted ($P < 0.05$)
H5: The means of final bindings at same incubation time and temperature with different adsorbent concentration are not equal.	b-f, h-l	Accepted ($P < 0.05$)

Lowercase letters represent the percentage of binding at specific concentrations of shells, incubation time, and incubation temperature as defined in Table 7

Fig. 2 Effect of adsorbents concentration increase on AFM1/chitin and AFM1/shell complex binding stability in milk before (initial) and after (final) five washes. Binding was determined after incubation of AFM1 (1 mL and 2 µg/L) with chitin and shrimp shells for 30 min at 37 °C. The formed complexes were washed five times with 1 mL of milk and the unbound AFM1 was quantified. The results are mean values from three experiments; *significant difference ($P < 0.05$) between initial binding; **significant difference ($P < 0.05$) between final binding. Error bars represent the SD (standard deviation)

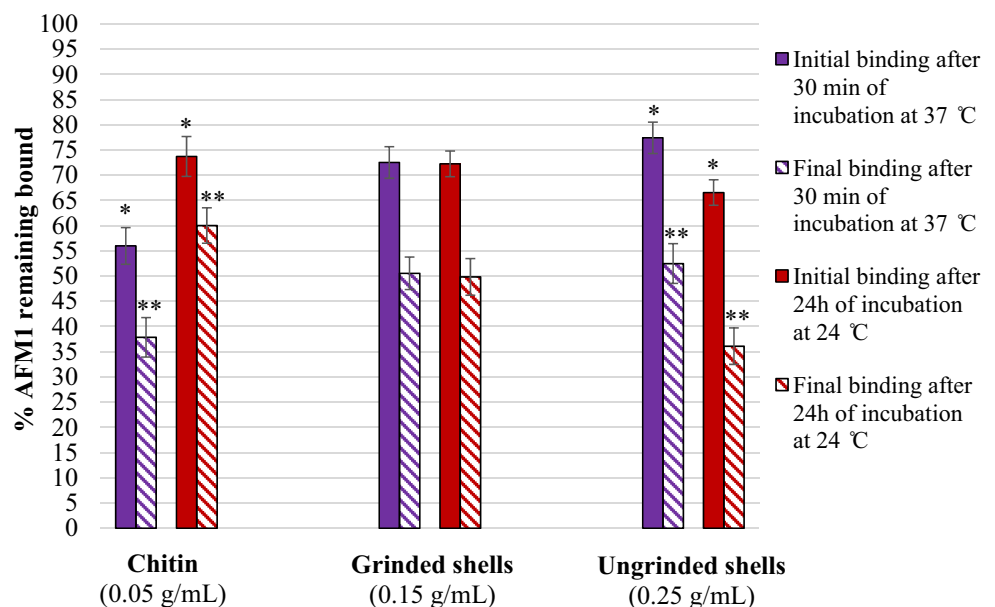


AFM1 to grinded shells in milk solution. These results were in accordance with the tests undertaken in PBS (Tables 3 and 4), where no significant ($P < 0.05$) binding difference was observed after increasing the incubation time of AFM1 with grinded shells from 30 min to 24 h. Therefore, this lack of significant binding changes in milk and in PBS are possibly due to a more direct contact between AFM1 and its binding site after grinding. The binding of ungrinded shells with AFM1 in milk decreased significantly ($P < 0.05$) after increasing the incubation period. In the theory of cold proteins, it was reported that proteins unfold upon increasing the temperature and fold when temperature decreases (Ben-Naim 2013). We could

speculate that this temperature decrease to 24 °C caused a possible increase in folding of shell proteins, making the entry and fixation of AFM1 on its binding site, possibly composed of chitin, difficult to achieve comparing to grinded shells.

Depending on the type of the adsorbent and the experimental conditions, different procedures can be adopted in order to eliminate AFM1 from beverage such as milk. For example, an adapted industrial agitator may be used for mixing treated shrimp shells or chitin with AFM1-contaminated milk to increase the bounded amount of AFM1. This procedure can be coupled with a filtration step to remove residual powder or shells. Due to its limiting factors such as its requirement of

Fig. 3 Effect of incubation times and temperatures on AFM1/chitin and AFM1/shell complex binding stability in milk before (initial) and after (final) five washes. Binding was determined after incubation of AFM1 (1 mL, 2 µg/L) with chitin and shrimp shells for 30 min (37 °C) and 24 h (24 °C). The formed complexes were washed five times with 1 mL of milk and the unbound AFM1 was quantified. The results are mean values from three experiments; *significant difference ($P < 0.05$) between initial binding; **significant difference ($P < 0.05$) between final binding. Error bars represent the SD (standard deviation)



energy for grinding, special treatment, and low selectivity during filtration step, grinded shells remain unfavorable for industrial applications. Thus, another proposed method for AFM1 decontamination consists of fixing effective quantities of treated ungrinded shells or chitin to form a membrane used to detoxify harmful AFM1 by passing contaminated liquids through or over it. In order to check any changes in the quality of milk after AFM1 decontamination, an organoleptic study of milk have to be conducted. The effect of additional parameters such as inoculum volume and agitation on AFM1 binding can be evaluated. It is noteworthy to note a binding of AFM1 to the used adsorbents in PBS. However, the potential use of *N*-acetylglucosamine, chitin, and treated crustacean shells as mycotoxin binders should be further studied in vitro and in vivo in order to elucidate its role. Accordingly, different industries might benefit from the use of these adsorbents including agro-food, medical, and pharmaceutical industries.

In conclusion, chitin or heat-treated shrimp shells were tested for their ability to bind AFM1. Increasing the concentration of these binders significantly increased the initial binding of AFM1 in milk and in PBS. In addition, increasing the incubation time positively affected the binding of AFM1 to chitin and ungrinded shrimp shells in PBS. Conversely, the binding of AFM1 to grinded shells was not affected by changes in incubation time and temperature indicating a stability and rapidity of binding. In milk, increasing the incubation time and decreasing the incubation temperature from 37 °C (30 min) to 24 °C (24 h) affected negatively the binding of AFM1 to ungrinded shells and positively to chitin. The partial reversibility of AFM1 binding observed after washing steps suggests the implication of electrostatic bounds such as hydrogen bonds and Van der Waals interactions. This work shed light on the ability of using novel adsorbents and techniques to bind and eliminate harmful AFM1 from milk and other liquids.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

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